INFECTION AND IMMUNITY, May 1996, p. 1750–1755 Vol. 64, No. 5
0019-9567/96/$04.00+0

A Cloned Major *Schistosoma mansoni* Egg Antigen with Homologies to Small Heat Shock Proteins Elicits Th1 Responsiveness

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Received 1 December 1995/Returned for modification 17 January 1996/Accepted 27 February 1996

In schistosomiasis mansoni, soluble egg antigens of the worm induce chronic T-cell-mediated granulomatous tissue responses. Since the first preparation of crude soluble egg antigen extract, a dearth of highly purified antigens has hampered the identification of granuloma inducer molecules. Here we report that a cloned 38-kDa egg polypeptide (r38) with homologies to small heat shock proteins is a strong immunogen. The recombinant and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis separated and eluted native 38-kDa (p38) polypeptides, used in microgram amounts and unaided by adjuvant, sensitized mice for a Th1-type immune response, with strong interleukin-2 (IL-2) and gamma interferon secretion but no IL-4 and IL-10 secretion. Extensive cross-reactivity between these two polypeptides was evident. This pattern was confirmed by reverse transcription-PCR that showed strong IL-2 and gamma interferon message expression but trace amounts of IL-4 message expression in r38-sensitized splenocytes. In mice, the polypeptide induced pulmonary mononuclear granuloma formation around antigen-coupled beads or worm eggs. We propose that the superior immunogenicity of r38 is linked to its relatedness to small heat shock proteins and that the 38-kDa polypeptide may induce the Th1 cytokine responses observed during the early development phase of the egg-induced granuloma.

Schistosomiasis mansoni is an helminth-induced disease of the tropics. The pathology of this disease is caused by disseminated worm eggs that lodge in the livers and intestines of infected hosts and evoke T-lymphocyte-mediated chronic granulomatous inflammation followed by irreversible fibrosis (2). In experimental animals, granuloma formation has been shown to be induced and elicited by soluble egg antigens (SEA) secreted by the miracidia within eggs (4–6, 18). Over the years several laboratories have isolated antigenic fractions from crude egg homogenates. A number of partially purified glycoproteins have been shown to possess serological, dermal, lymphocyte-stimulating, hepatotoxic, and granuloma-inductive properties (3, 7, 8, 15, 18, 19, 21–23, 25, 29, 36). However, the relative importance of the various fractions as granulomagenic agents remains unexplored. More recently, the differential responsiveness of acute- versus chronic-infection murine lymphocytes to a panel of SEA-derived fractions has been demonstrated. A 38-kDa fraction obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation was found to be egg stage specific, to elicit strong lymphokine production in vitro, and to induce granuloma formation in vivo during the acute stage of murine schistosomiasis (3, 22, 23).

Very few *Schistosoma mansoni* egg antigens have been sequenced (1, 20, 26, 28), and to date no attempt has been made to use defined egg antigens in characterization of the granulomatous immune response. The present study was initiated with the aims to clone a targeted *S. mansoni* egg antigen (the 38-kDa glycoprotein) with described strong immunogenic properties and to examine its biologic functions in vitro and in vivo. Using a full-length clone with an M_r of 38, a major egg antigen of *S. mansoni* with homologies to small heat shock proteins (28), we describe a typically Th1 pattern of cytokine production elicited in splenocytes from sensitized mice and from acute-infection animals. Moreover, the r38 polypeptide induced and elicited pulmonary granulomatous responses characterized by a predominantly mononuclear infiltrate.

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MATERIALS AND METHODS

Animals and parasites. Female CBA/J^k mice (Jackson Laboratories, Bar Harbor, Maine) 16 to 18 g, were used throughout this study. Mice were infected subcutaneously (s.c.) with 200 cercariae of a Puerto Rican isolate of *S. mansoni* for egg harvest or with 25 cercariae for cytokine production. Male LVG hamsters (51 to 60 g; Charles River, Boston, Mass.) were infected with 350 cercariae.

SEA preparation. SEA were prepared from homogenized ultracentrifuged eggs, freshly prepared from the intestines and livers of infected mice and hamsters, as previously described (6).

Isolation of p38 protein. The native 38-kDa antigen (p38) was prepared from SEA separated on an SDS–8% PAGE gel under nonreducing conditions, as described previously (21). The stained 38-kDa band was excised from the gel and electroeluted overnight. The effluents were pooled from consecutive elutions, desalted, and concentrated by using Centricon tubes (Amicon, Danvers, Mass.). The protein concentration of p38 was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.).

Cloning and purification of r38. *S. mansoni* mRNA was prepared by using a Quickprep mRNA purification kit (Pharmacia Biotechnology, Piscataway, N.J.). A cDNA library was constructed by inserting the cDNA unidirectionally into the Uni-ZAP vector at the *Eco*RI and *Xho*I sites (33). Plaques were screened with a polyclonal rabbit antiserum raised against the SDS-PAGE-separated, electroeluted 38-kDa fraction of SEA (23). Positive plaques were subcloned into the pGEX vector (Pharmacia) *Eco*RI and *Xho*I sites, which expressed an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible soluble glutathione *S*-transferase fusion protein. The soluble recombinant polypeptide (r38) was purified by using a bulk glutathione *S*-transferase purification module (Pharmacia). The sequence of r38 was analyzed by using the nonredundant PDB + GBupdate + GenBank + EMBLupdate + EMBL database program. Nucleotide sequencing of r38 showed 99% identity with the *S. mansoni* p40 egg antigen, with homologies to small heat shock proteins (13, 14, 28, 31, 32).

Sensitization of mice, preparation of cell populations, and generation of cytokine supernatants. Naive CBA/J^k mice were sensitized intraperitoneally (i.p.) or s.c. at the base of the tail with 1 μg of p38 or with 3 μg of r38. In some experiments, mice were sensitized s.c. with an emulsion of incomplete Freund's adjuvant (IFA) and 3 μg of r38. Spleens were removed under aseptic conditions 7 days after sensitization by established protocols (3). At 8 weeks after infection,

spleens were removed from at least three mice and single-cell suspensions were prepared, as described previously (21). Cytokines were measured in supernatants generated by incubating 3×10^6 splenic lymphocytes at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.3), 2 mM L-glutamine, 0.2 mM sodium pyruvate, 50 U of penicillin ml^{-1}, and 50 μg of streptomycin ml^{-1}. Cells were stimulated with SEA (10 μg ml^{-1}), p38 (1 μg ml^{-1}), or r38 (1 μg ml^{-1}). Supernatants were collected after 18 h for interleukin-2 (IL-2) and IL-4 determinations and after 48 h for IL-10 and gamma interferon (IFN-γ) measurements.

Measurement of cytokines. IL-2 levels were determined by comparing proliferation of the IL-2-dependent CTLL-20 cell line (a generous gift from Frank Fitch, University of Chicago, Chicago, Ill.) with a standard curve generated by using dilutions of recombinant murine IL-2 (generously donated by Cetus Corporation, Emeryville, Calif.). The specificity of this assay was confirmed by the complete abrogation of proliferative responses with anti-IL-2 monoclonal antibody (clone no. S4B6), kindly provided by DNAX Corporation, Palo Alto, Calif., and by the lack of responsiveness to high levels of IL-4 (up to 5,000 pM). IL-4 levels were similarly measured by using [^{3}H]thymidine uptake of the IL-4-dependent CT4S cell line (kindly provided by William Paul, National Institutes of Health, Bethesda, Md.) against dilutions of recombinant IL-4 (rIL-4) generously donated by Immunex Corporation, Seattle, Wash.). An anti-IL-4 monoclonal antibody (clone no. 11B11), provided by William Paul, confirmed that CT4S proliferative responses were absolutely dependent on the presence of IL-4. IL-10 was measured by an enzyme-linked immunosorbent assay (ELISA) using paired antibodies (clones SXC-1 and JES5-2A5) purchased from Endogen, Inc., Cambridge, Mass.; IFN-γ was also assayed by ELISA using paired antibodies (clones R46-A2 and XMG1.2) purchased from Pharmingen, San Diego, Calif. Biotinylated detecting antibodies were used in conjunction with an avidin-alkaline phosphatase conjugate. Color was developed by using nitrophenyl diamine diethanolamine, and the optical densities of wells were measured at 405 nm. Standard curves for each assay were made by using dilutions of recombinant cytokines (rIFN-γ was generously donated by Genentech; rIL-10 was purchased from Endogen). The threshold of sensitivity in ELISA was 45 to 50 pM for IL-10 and 20 to 25 pM for IFN-γ.

RT-PCR. Splenic lymphocytes from *S. mansoni*-infected mice or from mice sensitized with r38 were stimulated in vitro with 10 μg of SEA or 1 μg of r38. Total RNA extracted from cells removed after 18 h was used to determine IL-2 and IL-4 mRNA expression, while RNA from cells removed after 48 h was used for determinations of IL-10 and IFN-γ mRNA expression by reverse transcription-PCR (RT-PCR). RNA was extracted by using a Trizol total RNA isolation kit (Life Technologies, Gaithersburg, Md.). Total RNA was reverse transcribed into cDNA and amplified through 35 cycles by using an RT-PCR kit (Clontech, Palo Alto, Calif.) containing amplimers specific for murine IL-2, IFN-γ, IL-4, and IL-10. For the invariant message, control RT-PCR for β-actin was carried out. The primers for amplification were as follows: sense primer (sequence 105 to 124), GTGGGCCGCTCTAGGCACCA; and antisense primer (sequence 325 to 349), CGGTTGGCCTTAGGGTTCAGGGGGG (supplied by Genosys Biotechnologies, Inc., The Woodlands, Tex.).

Pulmonary granuloma formation. Mice were sensitized i.p. with 3 μg of p38 or r38 7 days before intravenous challenge with 2,500 *S. mansoni* eggs, prepared on the day of use, or with 2,500 Sepharose 4B beads to which p38 or r38 had been covalently linked (23). Four days after egg or bead challenge, lungs were removed and fixed in 10% buffered formalin. Fixed and paraffin-embedded lungs were sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Granuloma area measurements were made by using a Microcomp computerized photomorphometry software program.

Statistical analysis. Statistical analysis of our data was done by using the unpaired Student's *t* test.

RESULTS

Elicitation of predominantly Th1 immunoresponsiveness in splenocytes. As seen in cytokine production profiles, sensitization of mice with microgram amounts of native or recombinant 38-kDa polypeptide induced a Th1-type response. This was manifested by recall IL-2 and IFN-γ production by splenocytes when stimulated in vitro with p38. Sensitization and challenge experiments showed extensive cross-reactivity between p38 and r38 peptides. No IL-4 or IL-10 production was observed. Compared with the i.p. route, s.c. sensitization with r38 engendered a somewhat weaker Th1 response, which was greatly enhanced when the polypeptide was presented with IFA. Splenocytes of infected mice also responded to the p38 stimulus with strong IL-2 and IFN-γ production, indicating the presence of p38-reactive cells. However, IL-4 production was observed, which is consistent with Th0- or mixed Th1- and Th2-type cytokine reactivity (Fig. 1).

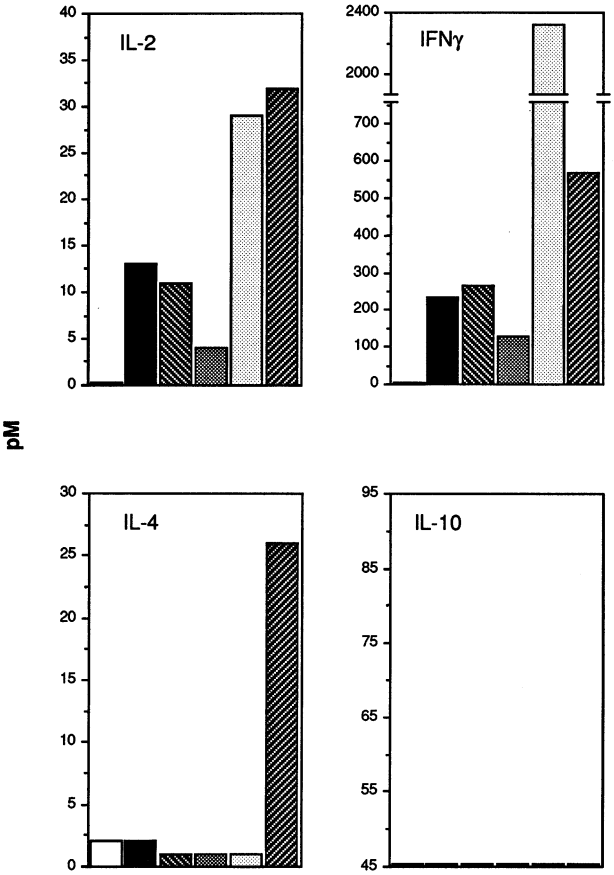

FIG. 1. Splenic lymphocytes from polypeptide-sensitized or infected mice exhibit a Th1-type cytokine secretion profile in response to in vitro stimulation with p38. Representative results, the means of two experiments, were determined through bioassay (IL-2 and IL-4) or ELISA (IFN-γ and IL-10). In each experiment, splenic lymphocytes were pooled from at least three mice. Route of sensitization: □, phosphate-buffered saline i.p.; ■, p38 i.p.; ◪, r38 i.p.; ▨, r38 s.c.; ▤, r38 + IFA s.c.; ▨, *S. mansoni* infection.

In vitro stimulation with r38 polypeptide elicited Th1-type cytokine responses comparable in magnitude with those obtained with p38. Sensitization via the s.c. route again induced a weaker cytokine response. However, r38-IFA-primed splenocytes responded with augmented IL-2 and IFN-γ responses to the homologous stimulus compared with the responses elicited by p38. Cross-reactivity between these two polypeptides was evident. Practically no IL-4 or IL-10 elicitation was observed with r38 in variously sensitized splenocyte cultures. Responsiveness was not dose dependent because i.p. immunization with 10-fold-higher doses of r38 (i.e., 30 μg) did not alter the cytokine production profile (data not shown). Although the polypeptide elicited low-level IL-4 production in splenic cultures of acute-infection mice, this was not significantly enhanced in the unstimulated control (data not shown). There was some modest IL-10 production (25 pM above the threshold of sensitivity) by acute-infection splenic cells stimulated by r38 (Fig. 2). In contrast, stimulation with SEA elicits three to eight times more IL-10 production (Fig. 3) (unpublished observations).

Crude SEA elicited levels of IL-2 production in p38- and r38-sensitized mice that were similar to those elicited by the homologous polypeptides. However, SEA stimulated IFN-γ production to a lesser degree in polypeptide-sensitized mice.

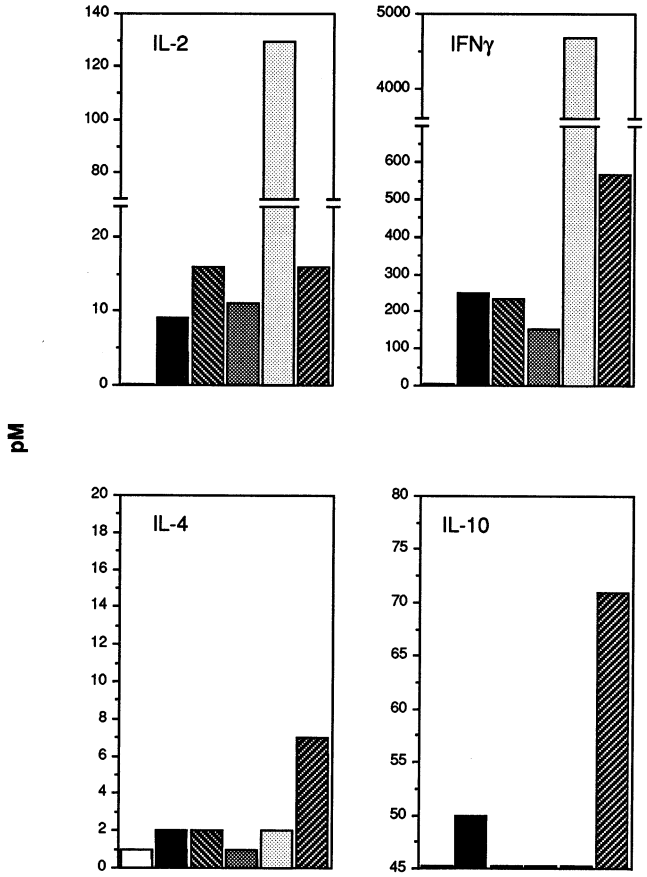

FIG. 2. Splenic lymphocytes from polypeptide-sensitized or infected mice exhibit a Th1-type cytokine secretion profile in response to in vitro stimulation with r38. Representative results, the means of two experiments, were determined through bioassay (IL-2 and IL-4) or ELISA (IFN-γ and IL-10). In each experiment, splenic lymphocytes were pooled from at least three mice. Route of sensitization: □, phosphate-buffered saline i.p.; ■, p38 i.p.; ◪, r38 i.p.; ▨, r38 s.c.; ▤, r38 + IFA s.c.; ▨, *S. mansoni* infection.

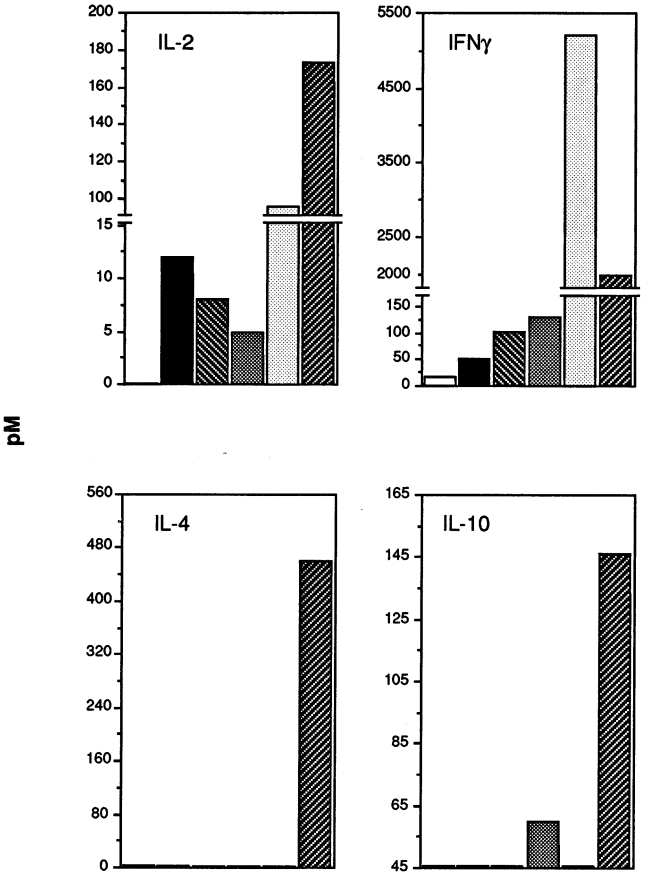

FIG. 3. Recall cytokine responses of splenic lymphocytes from polypeptide-sensitized or infected mice stimulated in vitro with crude SEA. Representative results, the means of two experiments, were determined through bioassay (IL-2 and IL-4) or ELISA (IFN-γ and IL-10). In each experiment, splenic lymphocytes were pooled from at least three mice. Cytokine production by unstimulated splenic lymphocytes of infected mice was as follows: IL-2, 0 pM; IFN-γ, 0 pM; IL-4, 4 pM; and IL-10, 16 pM. Route of sensitization: □, phosphate-buffered saline i.p.; ■, p38 i.p.; ◪, r38 i.p.; ▨, r38 s.c.; ▤, r38 + IFA s.c.; ▨, *S. mansoni* infection.

No IL-4 or IL-10 was elicited by SEA in p38- and r38-sensitized mice. Splenocytes of infected mice demonstrated Th0-type responsiveness, with strong IL-2, IFN-γ, IL-4, and IL-10 production to SEA-derived stimuli. The high levels of IL-4 and IL-10 produced are attributed to stimulation by fractions other than p38 (Fig. 3).

Confirmation of Th1 cytokine responsiveness by RT-PCR. RT-PCR confirmed the Th1-type responsiveness of splenocytes from sensitized or infected mice stimulated with r38. The r38-sensitized and -stimulated splenocytes demonstrated a Th1-type profile at the mRNA level, expressing strong IL-2 and IFN-γ messages but no IL-4 mRNA message (Fig. 4A). Although IL-10 mRNA was detected in both antigen-stimulated and unstimulated splenocytes, it was largely unaffected by antigenic stimulus. A similar pattern of Th1 cytokine gene expression was observed in splenic cells of acute-infection mice (Fig. 4B). Whereas p38 and r38 stimuli elicited strong IL-2 and IFN-γ gene expression but very weak IL-4 gene expression, the SEA stimulus elicited a Th0 or mixed Th1 and Th2 pattern that entailed significant IL-2, IFN-γ, IL-4, and IL-10 mRNA expression.

Pulmonary granuloma formation in sensitized mice. The immunogenicities of p38 and r38 were tested with respect to their granulomagenic activities. As Fig. 5 shows, granulomatous responses were induced in naive mice sensitized i.p. with either polypeptide without adjuvant. Responsiveness around live eggs was stronger than that around antigen-coated beads, most probably because of the slow, continuous release of antigen from eggs, providing higher antigen concentrations compared with those of covalently bound polypeptides. Extensive cross-reactivity was observed between p38 and r38; sensitization with one was sufficient to elicit granulomagenic activity against the other. The cellular composition of polypeptide-elicited granulomas was predominantly mononuclear, interspersed with the occasional neutrophil.

DISCUSSION

Very few *S. mansoni* egg-derived antigens have been obtained by molecular cloning (1, 20, 26, 28), and to date none has been examined for granuloma induction and elicitation. We targeted the egg antigen with an M_r of 38 for cloning because of its previously demonstrated antigenic potency (3, 21–23). Here we report that native electroeluted p38 and recombinant r38, presented i.p. or s.c. in the absence of adjuvant, induced predominantly Th1-type cytokine responsiveness (IL-2 and IFN-γ production) in splenic lymphocytes. This responsive-

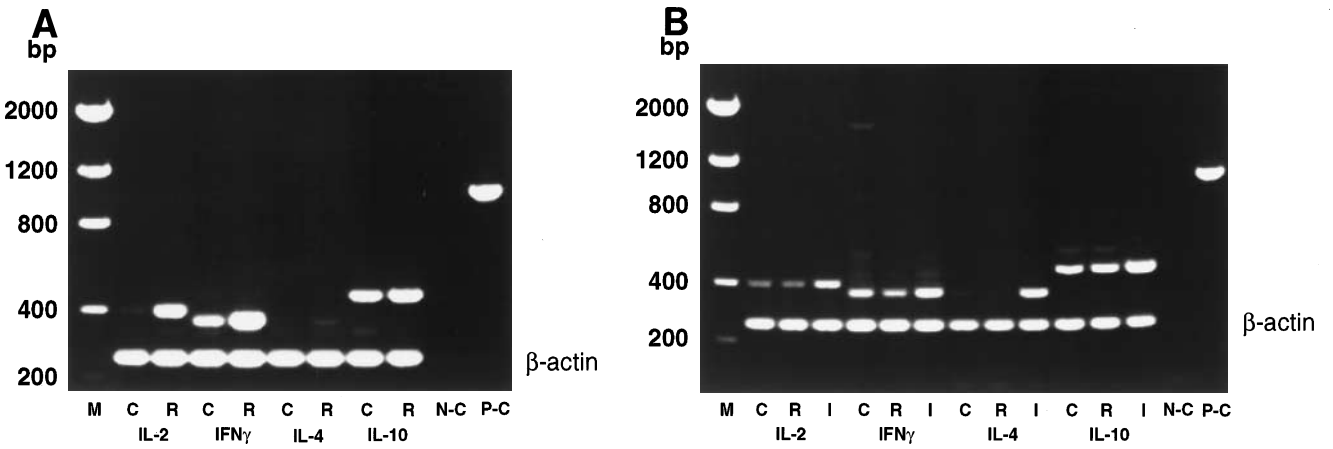

FIG. 4. RT-PCR assay of IL-2, IFN-γ, IL-4, and IL-10 mRNA expression in mouse splenic lymphocytes from r38-sensitized (A) and *S. mansoni*-infected (B) mice stimulated in vitro with r38 or SEA. Lanes: C, unstimulated sensitized cells; R, r38-stimulated cells; I, SEA-stimulated cells; M, 100-base ladder; N-C, negative control; and P-C, positive control (glucose-3-phosphate dehydrogenase DNA from the human pancreas). β-Actin was the invariant-message control.

ness, confirmed by RT-PCR amplification, was also manifest in pulmonary granuloma formation. The native and recombinant peptides showed similar antigenic potencies, as demonstrated by their extensive cross-reactivity. The induced Th1 responsiveness was not dose dependent and, except for enhancing the magnitude of IL-2 and IFN-γ secretion, was unaffected by the incorporation of r38 into IFA. It is noteworthy that splenocytes of acute-infection mice also exhibited the Th1 cytokine secretion pattern in response to native and recombinant polypeptides. This contrasted with the mixed Th1 and Th2 cytokine production profile (i.e., elevated IL-2, IFN-γ, IL-4, and IL-10 levels) in splenocyte cultures stimulated with unfractionated SEA. While IL-10 production in the supernatants of sensitized mice in response to these polypeptides was not detectable, IL-10 mRNA was clearly expressed. Much of this could be accounted for by IL-10 mRNA in unstimulated cells. The fact that no measurable cytokine was detected by ELISA would suggest either that cytokine secretion was below the threshold of sensitivity of the assay or that there was no translation of mRNA into protein product. Production of IL-10 in many delayed-type hypersensitivity systems has been described and appears to be only indirectly elicited, via the macrophage, in response to an antigenic stimulus (27). In schistosome egg-injected granulomatous lung tissue, IL-12 enhanced IL-10 mRNA expression, suggesting a linkage between IL-10 production and the Th1 pathway (37). Synchronous lung granulomas isolated from acute-infection mice also showed concurrent early production of IFN-γ and IL-10 cytokines (10). Indeed, recent experiments showed that granuloma macrophages produce significant levels of IL-10 (35). In the present experiments, the elicitation of IL-10 production by r38 in splenocyte cultures of infected mice may be attributable to a splenic adherent cell- or Th0 subset-mediated response.

On the basis of the pattern of cytokine production, several laboratories observed Th1-type responsiveness during the preoviposition stage of murine schistosomiasis mansoni. After oviposition, an egg antigen-induced shift to Th2-type responsiveness was described (16, 23, 30, 34). A similar shift occurred in the draining lymph nodes of mice s.c. sensitized with eggs and in intragranulomatous cytokine production during the growth phase of the primary lung egg or SEA-bead granuloma-elicited lesions (9–11). This changing pattern of cytokine production was confirmed by analysis of cytokine mRNA expression in the lungs of primary egg granuloma-bearing mice (37). In accord with those results, our laboratory has found that liver granuloma T cells also showed a strong Th1 response during the early growth phase (6 weeks), a mixed Th1 and Th2 response at peak evolution (8 weeks), and a predominant Th2 response at the modulated phase (20 weeks) of granulomas in infected mice (23). Moreover, Th0-type cells were cloned from granuloma lymphocytes, indicating their pluripotential activity in cytokine production (38).

Previously, we have shown that splenocytes of egg-primed mice responded within 4 days with IL-2 production to a p38-mediated stimulus and that this fraction elicited granuloma formation (3, 23). Thus, this polypeptide appears to be secreted immediately after egg deposition in tissues. Therefore, we propose that p38 within the SEA complex is one of the

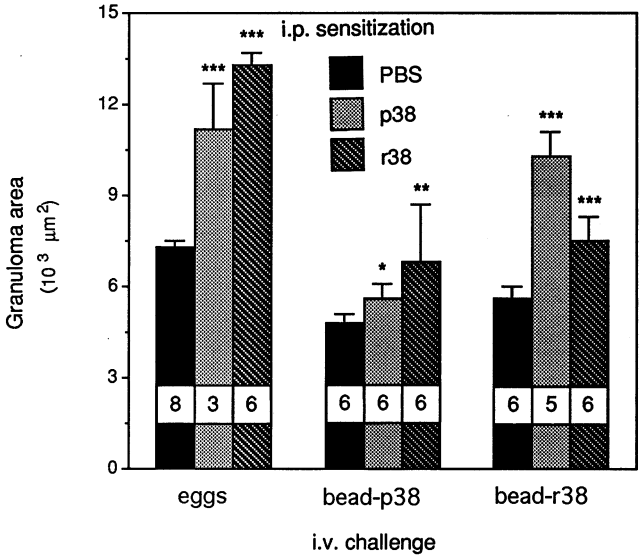

FIG. 5. Granulomatous responsiveness is elicited in mice sensitized i.p. with phosphate-buffered saline (PBS), p38, or r38 and challenged with *S. mansoni* eggs or bead-bound antigen. The total number of mice in each group is indicated. Asterisks indicate significant differences to the corresponding control group: *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test). The positive controls were four egg-sensitized, egg-challenged mice; granuloma area (10^3 μm^2), 27.8 ± 1.2. i.v., intravenous.

major immunogens responsible for induction of the Th1 responsiveness observed during the early growth of the granulomatous response. With the maturation of the granulomatous response, the Th1-type pattern is subsumed by an evolving strong Th2 response. Although the secretion of Th2 cytokines, especially IL-4, overwhelms p38-elicited IL-2 and IFN-γ production, we have shown that in the splenocytes of acute-infection mice, within the barrage there persists a significant p38-specific response.

The granuloma of murine schistosomiasis mansoni has been well characterized as a highly eosinophilic, macrophage-rich infiltrate, with lymphocytes constituting only about 10 to 15%. While a strong elicitor of Th1 cytokines, the antigen with an M_r of 38 also potentiated granuloma formation; however, unlike infected animals, mice immunized with r38 or p38 showed a predominantly mononuclear infiltrate around trapped eggs or bead-bound antigen. This is in accord with previous observations that demonstrated Th1 subset lymphocyte-mediated mononuclear granuloma formation (10–12).

The sequence of the gene encoding r38 was found to be identical to the published sequence of the gene that codes for p40, a major egg antigen of *S. mansoni* recognized serologically by over 70% of infected humans (28). This is the first study to examine the immunogenic properties of this recombinant polypeptide in mice. The published sequence of r38 has extensive homologies with the family of small heat shock proteins (13, 14, 28); such proteins found in bacteria and parasites act as immunodominant antigens evoking T- and B-cell responsiveness (24, 31, 32), eliciting Th1-type cytokine production in cloned T cells (17), and serving as carriers for synthetic peptides in adjuvant-free immunization (24). We propose that the strong immunogenicity of r38 for Th1 subset cells is vested in its molecular homologies with small heat shock proteins. Soluble, cloned r38 egg peptide will aid in the definition of cytokine responsiveness and granulomatous immunopathology and in better understanding of schistosomiasis mansoni.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-12913 from the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

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Editor: J. M. Mansfield